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1 *In vivo* visual screen for dopaminergic *Rab* ↔
2 *LRRK2-G2019S* interactions in *Drosophila*
3 discriminates *Rab10* from *Rab3*

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6 Stavroula Petridi^{1,2}, C. Adam Middleton¹, Chris Ugbo¹,
7 Alison Fellgett¹, Laura Covill^{1,3} & Christopher J. H. Elliott¹

8

9 1) Department of Biology and York Biomedical Research Institute, University
10 of York, York, YO1 5DD, UK

11

12

13 Present addresses:

14 2) School of Life Sciences, University of Warwick, Gibbet Hill Campus,
15 Coventry CV4 7AL, UK

16 3) Department of Haematology and Regenerative Medicine, Karolinska
17 Institutet, Blickavägen 8, Huddinge, Stockholm, Sweden.

18

19

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22

23 Correspondence to CJHE – Department of Biology, University of York, York,
24 YO10 5DD, UK.

25

26 Email: cje2@york.ac.uk

27 Tel: +44 1904 328654

28

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31

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34 CJHE revised the manuscript.

35

36 No conflicts of interest were perceived.

37

37 Abstract

38

39 LRRK2 mutations cause Parkinson's, but the molecular link from increased
40 kinase activity to pathological neurodegeneration remains undetermined.
41 Previous *in vitro* assays indicate that LRRK2 substrates include at least 8 Rab
42 GTPases. We have now examined this hypothesis *in vivo* in a functional,
43 electroretinogram screen, expressing each *Rab* with/without *LRRK2-G2019S*
44 in selected *Drosophila* dopaminergic neurons. Our screen discriminated Rab10
45 from Rab3. The strongest Rab/LRRK2-G2019S interaction is with Rab10; the
46 weakest with Rab3. Rab10 is expressed in a different set of dopaminergic
47 neurons from Rab3. Thus, anatomical and physiological patterns of Rab10 are
48 related. We conclude that Rab10 is a valid substrate of LRRK2 in
49 dopaminergic neurons *in vivo*. We propose that variations in *Rab* expression
50 contribute to differences in the rate of neurodegeneration recorded in
51 different dopaminergic nuclei in Parkinson's.

52

53

54 Introduction

55 Inherited mutations in *LRRK2* (*Leucine-rich-repeat kinase 2*) are a common
 56 cause of Parkinson's. A single amino-acid change, *G2019S*, increases LRRK2
 57 kinase activity (Greggio and Cookson 2009). This mutation results in a toxic
 58 cascade that kills *substantia nigra* dopaminergic neurons. However, the main
 59 steps in this pathological signalling pathway remain to be determined. Partly
 60 this is because LRRK2 is potentially a multi-functional protein, with kinase,
 61 GTPase and protein-binding domains. A diverse range of >30 proteins that
 62 might be phosphorylated by LRRK2 have been reported, suggesting it is a
 63 generalised kinase (Tomkins *et al.* 2018). However, several research teams
 64 have recently reported that LRRK2 is a more specific kinase, phosphorylating
 65 a range of Rab GTPases (Thirstrup *et al.* 2017; Steger *et al.* 2017; Fan *et al.* 2018;
 66 Liu *et al.* 2018; Jeong *et al.* 2018; Kelly *et al.* 2018).

67 Rabs are a plausible LRRK2 substrate leading to neurodegeneration, as they
 68 act as molecular switches interacting with a range of proteins (GEFs, GAPs
 69 and GDIs) regulating supply and delivery of cargo to membranes. Indeed
 70 many of the 66 Rabs in humans have been linked to neurodegenerative
 71 disorders (Kiral *et al.* 2018). Mutations in Rabs 29 and 39 cause Parkinson's
 72 (MacLeod *et al.* 2013; Beilina *et al.* 2014; Wilson *et al.* 2014). Biochemically, at
 73 least 8 seem to be directly phosphorylated by LRRK2 [Rabs 3, 5, 8, 10, 12, 29,
 74 35 and 43] (Steger *et al.* 2017). However, it is not clear which of the more than
 75 60 Rabs are actually phosphorylated *in vivo*. In mammals, analysis of the role
 76 of the Rabs is complex because individual Rabs may have similar, or even
 77 compensatory functions, which may differ by tissue (Chen *et al.* 2012; Kelly *et al.*
 78 *et al.* 2018). The situation is simpler in the fly, because there are fewer Rabs -
 79 only 23 mammalian orthologs. Here, we use a *Drosophila* screen to assess the
 80 link from LRRK2 to Rabs *in vivo* using the *Tyrosine Hydroxylase* (*TH*) GAL4 to
 81 achieve dopamine specific expression. UAS-*LRRK2-G2019S* (Liu *et al.* 2008)
 82 was driven with and without each *Rab* gene (Zhang *et al.* 2006).

83 We measured a visual phenotype using the SSVEP (Steady State Visual
84 Evoked Potential). Although the outer structure of the eye differs markedly
85 between flies and mammals, the retinal circuitry is highly similar (Cajal and
86 Sanchez 1915; Sanes and Zipursky 2010) – importantly both contain
87 dopaminergic neurons. In the human, the retinal dopaminergic neurons die in
88 Parkinson’s (Harnois and Di Paolo 1990), while in the *TH>G2019S* model of
89 Parkinson’s, the retina has visual deficits, including neurodegeneration
90 (Hindle *et al.* 2013; Afsari *et al.* 2014; West *et al.* 2015a). We can now use the
91 ability of the SSVEP assay to separate and quantify the response of the
92 photoreceptors and lamina neurons to go beyond measuring
93 neurodegeneration, but to test for a synergistic interaction of a Parkinson’s
94 related gene with potential substrates. Notably, we can do this *in vivo* in
95 young flies before degeneration has set in.

96 We determined that, *in vivo*, Rab10 has the strongest synergy with LRRK2-
97 G2019S, Rab3 the weakest. We validated the physiological results by showing
98 differences in the expression of *Rab10* and *Rab3* in visual dopaminergic
99 interneurons.

100 **Materials and methods**

101 **Flies** (*Drosophila melanogaster*) were raised and manipulated according to
102 standard fly techniques. Fly stocks are listed in Table 1. Crosses were raised at
103 25 °C on a 12:12 light-dark cycle. On the day of emergence, female flies were
104 placed in the dark at 29 °C.

105 **Screen design:** Virgins from the *TH-GAL4*, or from a *TH-GAL4::UAS-LRRK2-*
106 *G2019S* (*THG2*) recombinant were crossed with males carrying *UAS-Rab*, for
107 each of the *Rabs* that are homologous to those of mammals.

108 The principle of the SSVEP screen is shown in Fig. 1. The visual response of
109 flies stimulated with a flickering blue light was recorded. Young, 4-12 hour
110 old, PD-mimic flies show visual hyperexcitability, particularly in the lamina

neurons (Afsari *et al.* 2014; Himmelberg *et al.* 2017). This includes the *THG2* flies. As they age, the visual response gets weaker and vanishes by 28 days. We therefore chose to test flies aged for 24-36 hours (1 day) or 1 week - between the time at which *G2019S* expression results in hyperexcitability and the time at which degeneration is first noted. At these time points, the mean visual response of dark-reared *THG2* flies was similar to the *TH/+* controls.

Sample test for synergy: We test for an interaction between *Rab7* and *G2019S* in dopaminergic neurons as follows: we compare flies expressing both *Rab7* and *G2019S* transgenes (*THG2 > Rab7*) with flies expressing just one transgene (*TH > Rab7* or *THG2*) and control flies with no transgene expression (*TH/+*) (Fig. 1F). The average visual response of *TH > Rab7* and *THG2* flies is very similar to the control flies - there is no mean difference for either the photoreceptors or lamina neurons. We do note that the *THG2* flies have a larger variability than the *TH/+* flies, particularly in the lamina neurons (Fig. 1F). However, in flies with dopaminergic expression of both *G2019S* and *Rab7*, the photoreceptor and lamina neuron responses were much increased (4.1x and 8.8x, both $P < 0.001$). This demonstrates that dopaminergic neurons with both *Rab7* and *G2019S* have a synergistic hyperexcitable visual phenotype.

SSVEP preparation: At the required age, flies were prepared for SSVEP measurements using a pooter and nail polish to secure them in the cut-off tip of a pipette tip, without anaesthesia (Fig. 1B). Each fly was presented 5 times with a set of 9 flickering stimuli. In each stimulus, the average light intensity was the same, but the amplitude of the flicker was adjusted from 10 to 100%, giving a range of contrasts. Sample stimuli are shown in Fig. 1C. Offline, the Fast-Fourier Transform was applied to the responses, to separate the first harmonic (1F1), due to the photoreceptors from the second harmonic (2F1), due to the lamina neurons (Fig. 1D). Other harmonics present in the data were not analysed. For these first two harmonics, we plotted the contrast response function for each fly (Fig. 1E) and determined the best response of

141 that fly. This allowed us to determine the average visual performance for each
142 cross (Fig. 1F). This data pipeline is the same as that devised by Afsari *et al.*
143 (2014), but using an Arduino Due to generate the stimuli and record the
144 responses instead of a PC. Data were analysed in Matlab, Excel and R. Full
145 code at <https://github.com/wadelab/flyCode>.

146 **Immunocytochemistry** was performed as described recently (Cording *et al.*
147 2017). Tyrosine hydroxylase was detected with Mouse anti TH Immunostar
148 (22941, 1:1000). Fluorescent reporters (nRFP, eIf-GFP) were expressed in
149 dopaminergic neurons using the *TH*-GAL4. Images were prepared for
150 publication using ImageJ; original images are available on request.

151 **Western blots for EYFP**, encoded in each Rab transgene were made from
152 non-boiled fly head lysates, run on Novex pre-cast mini gels (NuPAGE 4-12%
153 Bis-Tris Gels, NP0322BOX, Thermo Scientific) in 1 × MOPS buffer and
154 transferred onto PVDF membranes using a Hoefer wet transfer tank (TE22) at
155 100V for 1 hr. Membranes were probed with Guinea pig anti-GFP (Synaptic
156 Systems, 1:1000). For detection of LRRK2 protein, boiled lysates were run on
157 4-20% Mini-PROTEAN TGX Precast gradient gels and transferred using the
158 same method. Membranes were probed with anti-LRRK2 (Neuromab, clone
159 N241A/34, 1:1000). α -drosophila synaptotagmin was used as a loading
160 control (West *et al.* 2015b). Densitometric analysis was carried out using
161 ImageJ.

162 **Statistics** were calculated in R, with the mean \pm SE reported by error bars or
163 median \pm interquartile range in box plots. Post-hoc tests were calculated for
164 ANOVA using the Dunnett test.

165 **Data Availability Statement:** Data tables (Excel sheets) and R code are open
166 access on GitHub:
167 https://github.com/wadelab/flyCode/tree/master/analyzeData/fly_arduino/G3
168 . Raw images and SSVEP traces are available on request. No new
169 reagents are described.

170 Results

171 *A visual expression screen identifies that Rab10 has the strongest genetic interaction*
 172 *with LRRK2-G2019S; Rab3 the weakest*

173 In order to identify the Rabs which show a strong synergy with *LRRK2-*
 174 *G2019S* we compared the increase in visual response due to expression of the
 175 *Rab* by itself (X in Fig. 1F, X-axis in Fig. 2A) against the further increase in
 176 visual response when both *Rab* and *G2019S* are expressed (Y in Fig. 1F, Y-axis
 177 in Fig. 2A). This plot places the Rabs along a spectrum, from those that
 178 interact synergistically with *G2019S* (top left) to those with a little or no
 179 interaction (bottom right). Thus, for some Rabs (10, 14, 27, 26) expression of
 180 both *G2019S* and the *Rab* in dopaminergic neurons leads to a big increase in
 181 the lamina neuron response. Interestingly, these Rabs have little effect when
 182 expressed alone. The converse is also true: for the *Rabs* with the biggest effect
 183 (3, 32, 1), adding *G2019S* has no further effect. This is true for both
 184 components of the SSVEP signal – that from the lamina neurons is higher, but
 185 parallel to the photoreceptor signal.

186 We wanted to examine which factors controlled this synergy. A number of
 187 hypotheses have been put forward in the *LRRK2/Rab* literature. First, Rabs
 188 previously linked to Parkinson's (Shi *et al.* 2017), either through population
 189 studies or through potential actions with Parkinson's-related genes, generally
 190 have a stronger response to *G2019S* than others (Fig. 2Bi). Indeed, the Rab
 191 furthest above the regression line is one that causes Parkinson's, *Rab39*
 192 (Wilson *et al.* 2014). Next, we tested if Rabs with a high degree of phylogenetic
 193 similarity clustered systematically, but did not find any difference (Fig. 2Bii).
 194 Then we examined where, on our spectrum, the Rabs phosphorylated *in vitro*
 195 [3, 5, 8, 10, 12, 29, 35 and 43] might lie. There is no close homolog for Rabs 12,
 196 29 or 43. Rabs 3, 5 and 8 are on the right of the spectrum, Rab35 in the middle
 197 and Rab10 on the top-left, so no clear pattern emerges. The *in vitro* data
 198 suggest that *LRRK2-G2019S* preferentially phosphorylates Rabs with Thr
 199 rather than Ser at the active site (Steger *et al.* 2016), but this is not evident from

the spectrum. Neither *in vitro* evidence for phosphorylation of the Rab by LRRK2, nor the amino-acid at the active site affects the regression (Fig. 2Biii,iv). We also noted that, in cell based assays, LRRK/Rab interactions have been noted at mitochondria (Wauters *et al.* 2019), lysosomes (Eguchi *et al.* 2018) or Golgi (Liu *et al.* 2018). Thus, we tested if the ‘main’ organelle associated with the Rab (Banworth and Li 2017) affected the position of a Rab on the spectrum, but found no sign that this affected the regression (Fig. 2Bv). However, the Rabs placed in the middle of the spectrum [2, 6, 9, 18] are linked to traffic in the Golgi or ER-Golgi.

Thus, the relationship between visual impact of *Rab* and impact of *Rab* + *G2019S* identifies 10, 14, 27 as having the strongest synergy. This holds for the responses of both photoreceptors and lamina neurons, with the same Rabs found clustered at each end of the spectrum.

The Rab10/G2019S interaction enhances neuronal signalling

Normally, flies with more excitable photoreceptors activate the lamina neurons more strongly, though there is some adaptation. The SSVEP response can be decomposed into two components – 1F1 and 2F1, corresponding to activity in the photoreceptors and lamina neurons respectively. This allows us to test the physiological relationship between the photoreceptors and lamina neurons, and to see if any Rab disrupts the retinal neuronal circuitry. Generally, as the photoreceptor response increases, so does the lamina neuron response (Fig. 3). This relationship is remarkably similar in young (day 1) and older (day 7) flies. However, there is a one marked outlier, *Rab10*, where the lamina neuron response at day 1 is ~5 times the value expected from the regression, and at day 7 is substantially below the line. Thus, in young *THG2* > *Rab10* flies there is much greater neuronal activity than expected, but in 1-week old *THG2* > *Rab10* flies we observe reduced activity, suggesting neurodegeneration has begun. Young and old *Rab3* flies lie on the regression, close to the origin, very different from *Rab10*.

229 Thus our screen highlights a major difference between two of the Rabs that
230 are phosphorylated in vitro: *Rab3* expression in dopaminergic neurons has a
231 big increase in visual sensitivity, but no further effect when *G2019S* is added,
232 whereas *Rab10* expression has little effect by itself, but a massive effect in
233 young flies with *G2019S*.

234 *Why might G2019S interact so strongly with Rab10 but have no effect on Rab3?*

235 As LRRK2 is a human protein, and the Rabs we expressed were native
236 *Drosophila* proteins, one possibility is that the fly and human Rabs are
237 sufficiently different that LRRK2-G2019S can phosphorylate fly *Rab10* but not
238 fly *Rab3*. This seems very unlikely as the h*Rab3* / d*Rab3* and d*Rab10* /
239 h*Rab10* sequences are very similar, indeed they are identical over the GTP
240 binding domain and LRRK2 phosphorylation sites (Fig. 4). A second
241 explanation for the difference between *Rab10* and *Rab3* is that the *Rab* and/or
242 *G2019S* is not expressed to the same extent. Western Blots of *THG2* > *Rabs 3*,
243 39 or 10 were probed for LRRK2, and compared with *THG2*. Essentially the
244 same level of protein was measured (Fig. 5A). This is not unexpected, as each
245 cross contains the same GAL4 and UAS-LRRK2-G2019S constructs. A second
246 set of blots were probed for EYFP, as each of the UAS-*Rab* lines carries an
247 EYFP fusion. This showed that the levels of *Rab10* and *Rab39* were similar,
248 though *Rab3* was less at about 50% (Fig. 5B). The reduced level of *Rab3* may
249 arise from the different insertion site, or from a more rapid breakdown during
250 synaptic signalling. The differences in level of *Rab* proteins are not sufficient
251 to explain the physiological differences.

252 We therefore wondered if the stronger synergy between *G2019S* and *Rab10*,
253 compared with *Rab3*, might result from a difference in the anatomical
254 distribution of the Rabs (along with their GEFs, GAPs and effectors) among
255 fly dopaminergic neurons.

256 *Rab10 and Rab3 are found in different dopaminergic neurons*

257 The fly visual system is innervated by three kinds of dopaminergic neurons
258 (Hindle *et al.* 2013), the MC neurons in the medulla, and two type of PPL
259 neurons, which innervate either lobula or lamina respectively. These, and the
260 other clusters of dopaminergic neurons, are reliably marked by α -TH
261 antibody, which binds in the cytoplasm.

262 To examine the overall distribution of Rab10, we used *Rab10*-GAL4 (Chan *et*
263 *al.* 2011) to express either a RFP which strongly localises to the nucleus, or a
264 GFP with mainly nuclear localisation. These fluorescent constructs have two
265 advantages: (i) they provide a reduced background compared with
266 membrane localised reporters, and (ii) the nuclear fluorescence is contained
267 within the cytoplasmic signal from α -TH, reducing the problems of
268 determining co-localisation.

269 Only a small proportion of CNS neurons are Rab10 positive (Fig. 6). We find
270 that some (by no means all) dopaminergic neurons are Rab10 positive (Fig.
271 6A,B). Even within a cluster, we only detect Rab10 in some neurons; in other
272 neurons in the same cluster Rab10 is undetectable (e.g. PAL, PPL2ab, PPM3
273 and PAM). The individually identifiable neurons (TH-VUM, TH_AUM, the
274 DADN pair, and T1 pair) were consistently clearly marked. However, in two
275 clusters we saw no evidence for *Rab10* driven fluorescence (PPL2c and
276 PPM1/2).

277 When we used *Rab3*-GAL4 to drive the same RFP/GFP almost all the neurons
278 were marked (Fig. 6 C, Di). This includes the majority of the dopaminergic
279 neurons, including all the PPL1 (Fig. 6 Dii-iv) and PPL2 neurons.

280 The MC neurons in the optic lobes were not marked in either the Rab10 or
281 Rab3 experiments (Fig. 6 Aiv, Biv), though other Rab10 / Rab3 positive
282 neurons are present nearby. Since the MC neurons do not generally stain well
283 with GFP (Nassel and Elekes 1992; Hindle *et al.* 2013)), we tested if the MC
284 neurons were detected with *TH*-GAL4 > *nRFP*. This marked all the neurons
285 highlighted by α -TH, except the MC neurons. The MC neurons do express

TH, along with other genes linked to dopamine - *ddc* (*dopa decarboxylase*), *Vmat* (*vesicular monoamine transporter*) and *DAT* (*dopamine transporter*) (Davis *et al.* 2020) so are genuinely dopaminergic. The MC neurons are one of three kinds of Medulla intrinsic neurons that express *Rab10* at high levels, while all the optic lobe neurons (including MC) have high expression of *Rab3* (Davis *et al.*, 2020, extended data at <http://www.opticlobe.com/>).

Thus, we conclude that some of the dopaminergic neurons in the visual system are *Rab10* positive. These are some of the PPL cluster that innervate the lobula or project to the lamina, and the MC neurons in the medulla. All dopaminergic neurons are *Rab3* positive.

Differences in the loss of dopaminergic neurons between neuronal clusters

Drosophila models of Parkinson's have consistently shown loss of dopaminergic neurons with age when *LRRK2*, *α-synuclein* or *parkin* were manipulated. For *LRRK2*, most of the published information is for the Parkinson's-causative mutations *G2019S* or *I2020T*, driven by *DDC-GAL4*. This expresses in the dopaminergic and some serotonergic neurons. By 6-7 weeks (about two-thirds of the fly lifespan), about 25-50% of the dopaminergic neurons have been lost. For each cluster, there is quite a spread of the data (Fig. 7), which is most likely due to differences in the food used to feed the flies or the genetic background (Lavoy *et al.* 2018; Chittoor-Vinod *et al.* 2020). However, overall, the PAL cluster is much less susceptible to cell loss than the PPL1, PPL2, PPM1/2 or PPM3 clusters.

Discussion

Rab10 shows a strong synergy with *LRRK2-G2019S*.

The key observation from the screen was that two of the Rabs suggested to be substrates of *LRRK2 in vitro* behave quite differently *in vivo*, in a physiological response to expression in dopaminergic neurons. *Rab10* shows a strong

314 synergy with *G2019S*; Rab3 none. The existence of (*Drosophila*) Rab10 in the
 315 tyrosine hydroxylase positive neurons controlling vision (MC and PPL2ab
 316 neurons) argues that LRRK2 might indeed phosphorylate dRab10 directly.
 317 Thus our *in vivo* results both support the *in vitro* (biochemical and cell culture)
 318 data in which LRRK2 directly phosphorylates hRab10 (Thirstrup *et al.* 2017;
 319 Steger *et al.* 2017; Fan *et al.* 2018; Liu *et al.* 2018; Jeong *et al.* 2018; Kelly *et al.*
 320 2018). It also implies that the MC / PPL2ab cells contain Rab10 effectors
 321 which interact with phospho-dRab10. The results of this will include changes
 322 to the cellular homeostasis and physiological responsiveness of dopaminergic
 323 neurons. One possible physiological outcome is that Rab10 phosphorylation
 324 reduces retinal dopamine release onto the photoreceptors. This will increase
 325 the amplitude and speed of the photoreceptor response (Chyb *et al.* 1999).
 326 Dopamine may also affect the lamina neurons, and third order MC cells, but it
 327 remains to be determined if they have dopamine receptors. It is also possible
 328 that p-Rab10 modulates the release of co-transmitters or growth factors from
 329 dopaminergic neurons.

330 A unique feature of the screen is that when *G2019S* and *Rab10* are expressed
 331 together the lamina neuron response is much bigger than that predicted from
 332 the photoreceptor response. This might arise from the unusual double role of
 333 Rab10 – in both exo- and in endo-cytosis (Larance *et al.* 2005; Glodowski *et al.*
 334 2007; Chua and Tang 2018). The best defined role of Rab10 in exocytosis is in
 335 adipocytes, as part of the insulin-stimulated release of GLUT4 vesicles, linked
 336 to AS/160 (see for review (Jaldin-Fincati *et al.* 2017)). In endocytosis, the
 337 effects of Rab10 are mediated through a different pathway, including the
 338 EHBP1-EHD2 complex. In the follicle cells of *Drosophila*, *ehbp1* expression and
 339 knockdown phenocopy Rab10 manipulations (Isabella and Horne-Badovinac
 340 2016), while EHBP1 was also identified by a systematic proteomic analysis as
 341 indirectly phosphorylated by LRRK2 in HEK293 cells (Steger *et al.* 2017) and a
 342 lysosomal assay (Eguchi *et al.* 2018). The phosphorylation of Rab10 by LRRK2
 343 may switch its effector, and so activate a different pathway.

344 *A spectrum of Rab ↔ G2019S interactions in vision*

345 Our screen placed the Rabs along a spectrum, ranging from those with a
346 strong synergy with *G2019S* to those which had a strong effect when
347 expressed by themselves.

348 Among the Rabs which show little synergy with *G2019S* but have strong
349 visual effect are 1, 3, 5, 6 and 11. Two of these Rabs [3,5] are phosphorylated
350 by LRRK2 *in vitro* (Steger *et al.* 2017), but neither synergise with LRRK2-
351 *G2019S* in the visual assay. Our data suggest Rab3 is not a major substrate of
352 LRRK2-*G2019S* in these dopaminergic neurons, possibly because Rab3 is
353 located synaptically. This may be far from LRRK2 at the trans-Golgi network
354 (Liu *et al.* 2018). The difference between Rab3 and 10 (at opposite ends of our
355 spectrum) is notable because *in vitro* mammalian cell assays have highlighted
356 similar roles of Rabs 3 and 10 in lysosome exocytosis, (Encarnação *et al.* 2016;
357 Vieira 2018).

358 Rabs 10, 14 and 27 have the strongest synergy with *G2019S*, though by
359 themselves they have little effect on visual sensitivity. Like Rab10, Rabs 14
360 and 27 have defined roles in exocytosis (Larance *et al.* 2005; Ostrowski *et al.*
361 2010).

362 Some Rabs are in the middle of the spectrum [2, 6, 9, 18], with a 2-3 fold
363 increase in visual response when the *Rab* is expressed alone, and a further 2-3
364 fold increase when both *Rab* and *G2019S* are expressed. These Rabs have been
365 linked to the Golgi, or to Golgi-ER traffic (Banworth and Li 2017). Thus a
366 cellular phenotype parallels the physiological response.

367 Our observation that every *Rab* seems to have some effect on dopaminergic
368 signalling in the visual system goes some way to explain why many studies of
369 individual Rabs have demonstrated effects with LRRK2 ; Rab3a (Islam *et al.*
370 2016); Rab5 (Shin *et al.* 2008); Rab7 (Dodson *et al.* 2012); Rab29 (Beilina *et al.*
371 2014). Although cellular studies support binding of Rab29 to LRRK2 (Purlyte

372 *et al.* 2018), the closest fly homolog (Rab32) shows little synergy with G2019S
373 in our screen.

374 The availability of Rab transgenic flies facilitates screening in *Drosophila*.
375 Screens have identified key roles for Rab2 in muscle T-tubule development
376 (Fujita *et al.* 2017); Rabs 2, 7, 19 in loss of huntingtin (White *et al.* 2015), 1, 5, 7,
377 11 and 35 in the *Drosophila* renal system (Fu *et al.* 2017), Rab32 in lipid storage
378 (Wang *et al.* 2012) and Rab39 in tracheal formation (Caviglia *et al.* 2016). The
379 varied outcomes of these screens indicate the validity of the LRRK2-G2019S
380 ↔ Rab10 relationship reported here.

381 *Each dopaminergic neuron has its own palette of Rab expression*

382 Finally, we note that not all dopaminergic neurons are equally susceptible in
383 Parkinson's. A long-standing observation is that the dopaminergic neurons in
384 the VTA (ventral tegmental area) do not degenerate in the same way as those
385 in the *substantia nigra*. More particularly, even within the *substantia nigra* there
386 is a range of outcomes, with dopaminergic neurons in the *pars compacta* dying
387 more than those in the dorsal and lateral zones (Damier *et al.* 1999). The same
388 is true for the fly brain: the neurons in the PPM clusters degenerate more than
389 the PAL (though no data are available for the visual MC neurons). If
390 anything, our data suggest the clusters with less Rab10 have more
391 neurodegeneration. Previously, faster neurodegeneration has been ascribed
392 to increased cytosolic dopamine levels (Burbulla *et al.* 2017), to intracellular
393 effects of glutamate (Steinkellner *et al.* 2018), to increased calcium influx
394 (Guzman *et al.* 2010), to more action potentials (Subramaniam *et al.* 2014), or to
395 longer axons with more synapses (Pacelli *et al.* 2015). It has not escaped our
396 notice that faster degeneration in some neurons may be the result of their
397 different palettes of Rab proteins and their effectors.

398

399

Figure Legends

Fig. 1. SSVEP (Steady State Visual Evoked Potential Analysis) measures the contrast response function of the insect eye. A. The fly eye consists of ~800 ommatidia, each containing 8 photoreceptors. Their axons project to the lamina and medulla, where they synapse with the second- and third order neurons (lamina and medulla neurons). The medulla contains intrinsic dopaminergic neurons (MC, also called Mi15 neurons (Davis *et al.* 2020)), while some dopaminergic neurons from the CNS project to the lamina. B. Recording the fly visual response: A fly, restrained in a pipette tip, is illuminated with blue light from a LED, and the voltage across the eye is amplified and recorded. C. Repetitive stimuli given to the fly about a fixed mean light level evoke a contrast response increasing with the peak-peak excursion of the stimulus waveform. D. The response to a series of identical stimuli is analysed by the Fast Fourier Transform, and averaged. This shows a response at the stimulus frequency (1F1) and additional components at multiples of the input, notably twice the input frequency (2F1). Genetic dissection shows that the 1F1 component is mostly generated by the photoreceptors and the 2F1 by the lamina neurons (Afsari *et al.* 2014; Nippe *et al.* 2017). E. Plotting the amplitude of the 1F1 and 2F1 components against the stimulus contrast generates a CRF (Contrast Response Function), which differs from fly to fly. F. The averaged maximum CRF is dependent on genotype, with *THG2* (flies expressing *LRRK2-G2019S* in their dopaminergic neurons under the *Tyrosine Hydroxylase-GAL4, TH*) and *TH > Rab7* both showing a similar mean response to control flies (*TH/+*). However, flies expressing both *G2019S* and *Rab7* in their dopaminergic neurons (*THG2 > Rab7*) have a larger mean response than any other genotype, indicating synergy. The differences marked X (between the mean *TH/+* and *TH > Rab7*) and Y (between the mean *TH > Rab7* and *THG2 > Rab7*) are used as the X and Y axes of Fig. 2A. Box-plot representing median and interquartile range. Exact

432 genotypes and sample sizes: *TH*+/+, *TH*/*w*¹¹¹⁸, N= 7; *THG2*, *TH*::*G2019S*/*w*¹¹¹⁸,
433 N=11; *TH* > *Rab7*, N=11; *THG2* > *Rab7*, N=12.

434

435 **Fig. 2. Expression screen highlights *Rab10* with the strongest synergy with**
436 ***LRRK2-G2019S*, and *Rab3* as the weakest.** Each *Rab* was expressed in
437 dopaminergic neurons (using *TH*-GAL4) by itself (*TH* > *Rab*), or along with
438 *G2019S* (*THG2* > *Rab*) and the visual response measured after 24-36 hours
439 (labelled 1 day) or 7 days in the dark. A. ***Rab10* has the strongest synergy**
440 **with *G2019S*.** Relationship of *Rab* and *G2019S* showing their inverse
441 relationship. Rabs (3, 32, 1) which have a big effect on vision when expressed
442 on their own have little further consequence when *G2019S* is also expressed;
443 but other Rabs (10, 27, 14, 26) which have little visual impact on their own
444 have a strong synergy with *G2019S*. B. **An established role in Parkinson's is**
445 **the only factor that influences the inverse relationship between *TH* > *Rab***
446 **and *THG2* > *Rab*.** The LRRK2 ↔ *Rab* data in Fig. 2A are replotted here to test if it
447 is affected by factors that have been proposed to influence LRRK2 ↔ *Rab*
448 interactions. (i). Rabs previously linked to Parkinson's (Shi *et al.* 2017) have a
449 stronger *Rab* ↔ *G2019S* response than those which do not influence
450 Parkinson's, since a higher proportion of the magenta points lie above the line
451 (Fisher's exact test, P = 0.036). B(ii). Sensitivity is not linked to the phylogenetic
452 grouping of the fly Rabs (Zhang *et al.* 2006) . B(iii). Rabs usually have a serine
453 (Ser) or threonine (Thr) where they could be phosphorylated by LRRK2, though
454 *Rab40* has a histidine (His) (Zhang *et al.* 2006). Although a preference for LRRK2
455 to phosphorylate Rabs with a threonine was suggested by *in vitro* assays (Steger
456 *et al.* 2016), *in vivo* this is not detected. B(iv). Some Rabs are phosphorylated by
457 LRRK2 *in vitro* (Steger *et al.* 2017), but these Rabs are not more sensitive to
458 *G2019S* *in vivo*. B(v). The proposed main functional role of the *Rab* (Banworth
459 and Li 2017) does not affect the regression. Total flies: 1119, at least 9 for each
460 data point. Bars represent SE.

461

462 **Fig. 3. Standout role of *Rab10* with *G2019S* in neuronal signaling.** The
463 SSVEP response is split into two components, representing the

photoreceptors and lamina neurons (inset orange and purple). For each Rab, the increase in lamina neuronal signaling due to *G2019S* is plotted as a function of the photoreceptor signal. The increase in lamina neuron response is highly correlated with the response of the photoreceptors, with one outlying exception, *Rab10* at 1 day. Total flies: 1119, at least 9 for each data point. Bars represent SE.

Fig. 4. High sequence homology between *Drosophila* and human Rabs.

Comparison of fly and human Rab10 (A) and Rab3 (B) showing conservation in the GTPase domain and prenylated region. Also shown is the region which is phosphorylated *in vitro* by LRRK2, again highly conserved.

Fig. 5. Similar Expression of LRRK2-G2019S and Rab-GFP in dopaminergic neurons.

A. Co-expression of a *Rab-GFP* transgene does not affect the levels of *LRRK2-G2019S*. (i) Sample blot, (ii) Quantification of 3 replicates. B. Similar levels of Rab10 and Rab39, and less Rab3 when driven with *LRRK2-G2019S*. (i) Sample blot, (ii) Quantification of 3 replicates. wild-type is *CS/w⁻*, *TH/+* is *TH/empty vector*.

Fig. 6. Rab10 and Rab3 are located in different subsets of the dopaminergic neurons.

A, B. Rab10 is detected in some of the dopaminergic neurons that control vision (PPL1, Aii, Bii; PPL2 Aiii, Biii). Not all dopaminergic neurons, identified by a cytosolic α -Tyrosine Hydroxylase antibody (α -TH, green), are indicated by *Rab10-GAL4* expression of a strong nuclear RFP or the mainly nuclear eIf-GFP (magenta). The dopaminergic MC neurons in the visual lobes do not stain well with fluorescent reporters (Nassel and Elekes 1992; Hindle *et al.* 2013) and we could not detect *Rab10*-driven fluorescence (MC, Aiv, Biv, marked with grey in E). C, D. **Rab 3 is present in all dopaminergic neurons.** *Rab3-GAL4* driven nuclear RFP or eIf-GFP (magenta) marks most neurons, including nearly all that are dopaminergic (green). The PPL neurons not marked by *Rab10* expression are included (Dii-iv). E. Summary of the

495 expression pattern of (i) *Rab10* and (ii) *Rab3*. The MC neurons in the optic lobe
 496 (Nassel *et al.* 1988) are also called Mi15 neurons (Davis *et al.* 2020). Ai, Bi, Ci
 497 and Di: projection of confocal stacks through the whole CNS; Aii, Aiii, Bii,
 498 Biii, Dii-iv projections of confocal stacks through the cell groups,
 499 approximately marked in the whole CNS image; Aiv and Biv sections from a
 500 separate preparation to Ai and Bi. Data representative of at least nine brains
 501 (from at least 3 crosses), 3-7 days old. The *Rab3 > nRFP* flies were raised at 18
 502 °C to improve viability. Exact genotypes: +; *RedStinger4 nRFP/+*; *Rab10 Gal4/+*;
 503 or +; *RedStinger4 nRFP/+*; *Rab3 Gal4/+*; or +; *elf-4A3-GFP/+*; *Rab10 Gal4/+*; or +;
 504 *elf-4A3-GFP/+*; *Rab3 Gal4/+*;

505

506 Fig.7. Differences in neuron survival in dopaminergic clusters when an
 507 increased kinase mutation (*G2019S* or *I2020T*) is expressed with *DDC-GAL4*
 508 (which expresses in dopaminergic and serotonergic neurons) (ANOVA, 4,45
 509 df, $P < 0.002$). Data collected from (Liu *et al.* 2008; Ng *et al.* 2009; Xiong *et al.*
 510 2012; Angeles *et al.* 2014, 2016; Martin *et al.* 2014; Nucifora *et al.* 2016; Lin *et al.*
 511 2016; Sun *et al.* 2016; Basil *et al.* 2017; Marcogliese *et al.* 2017; Yang *et al.* 2018;
 512 Lavoy *et al.* 2018; Sim *et al.* 2019; Maksoud *et al.* 2019; Chittoor-Vinod *et al.*
 513 2020). Differences in the extent of degeneration within a neuronal cluster may
 514 be partially explained by differences in the composition of the fly food
 515 (Chittoor-Vinod *et al.* 2020).

516

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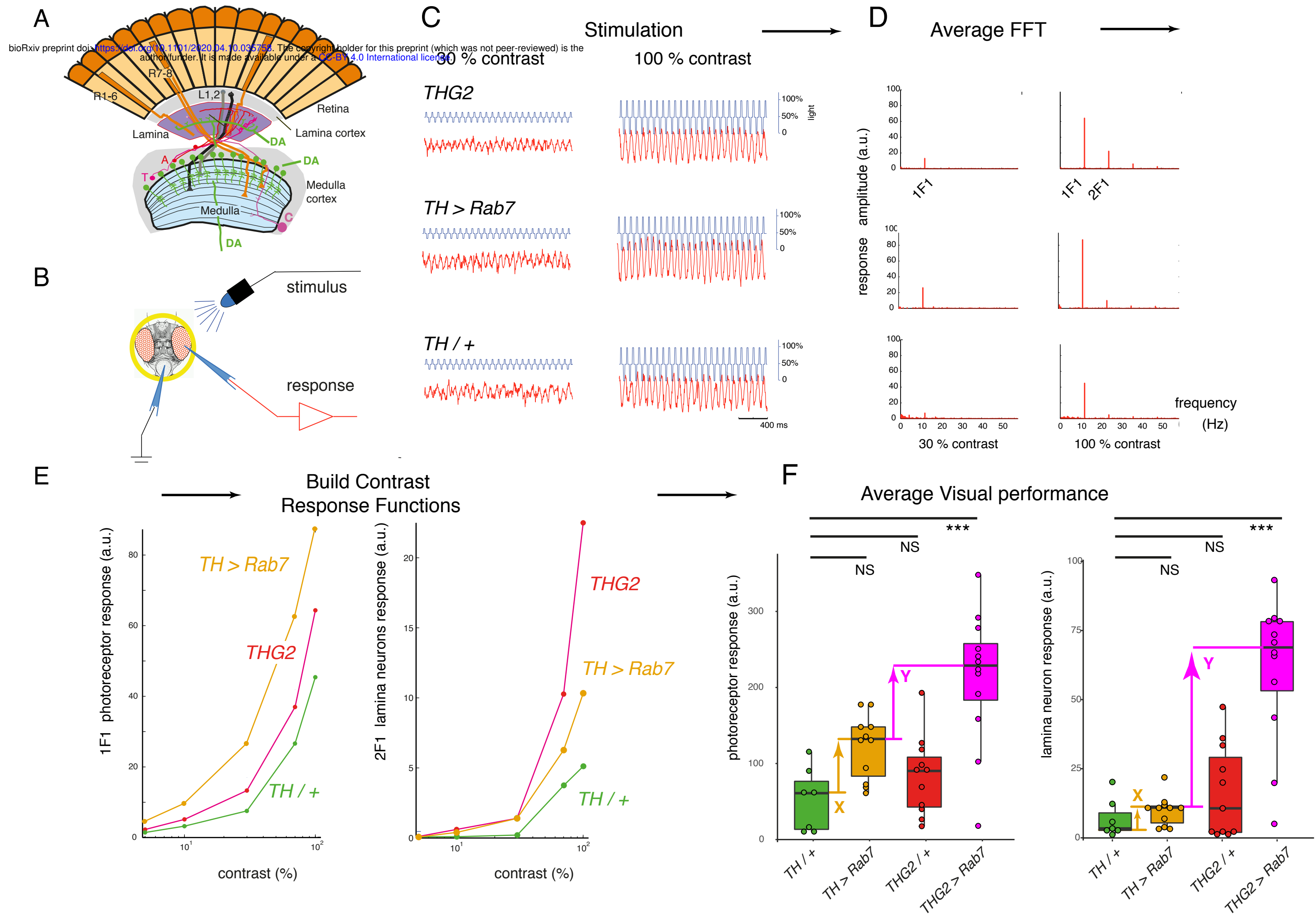
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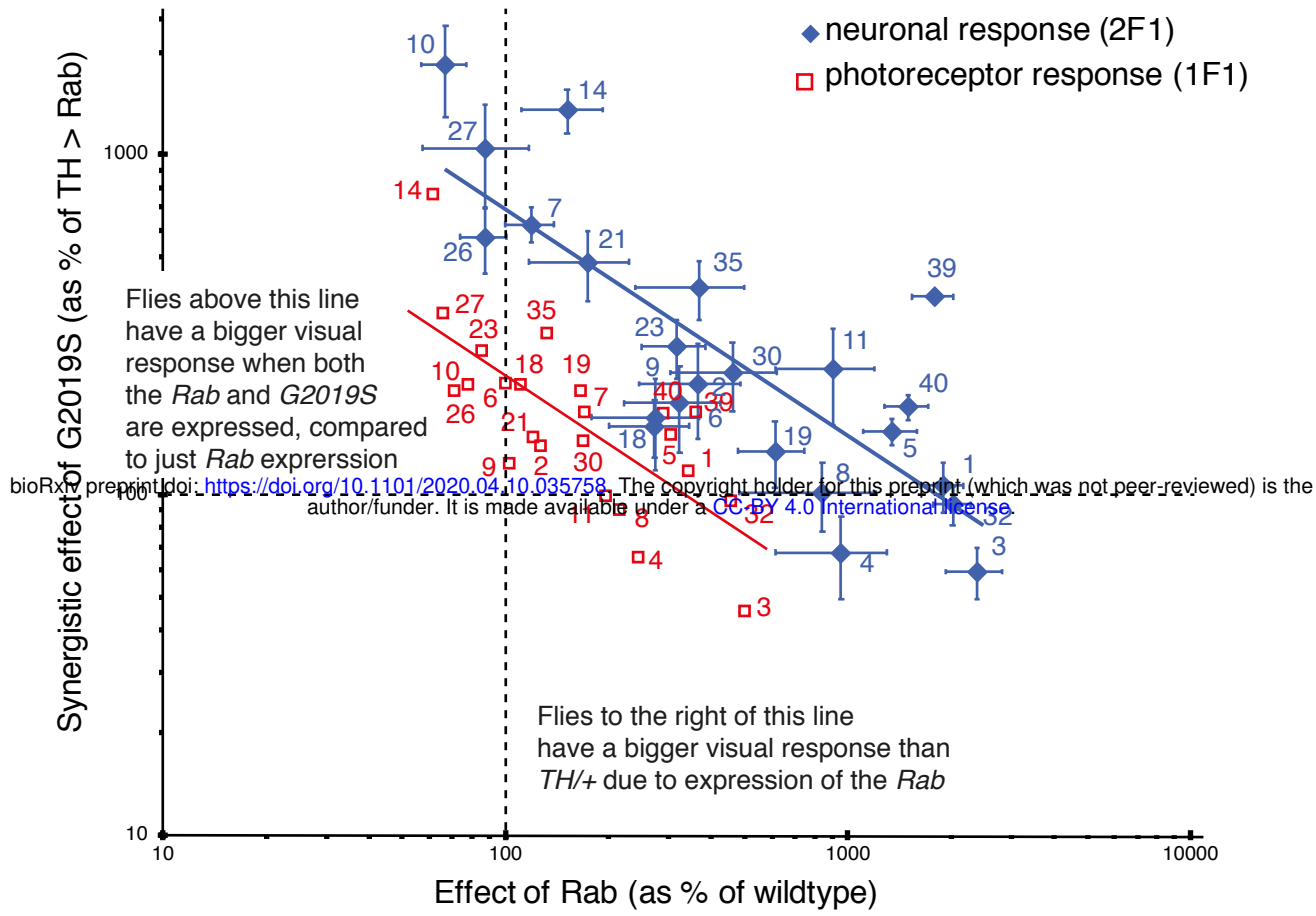
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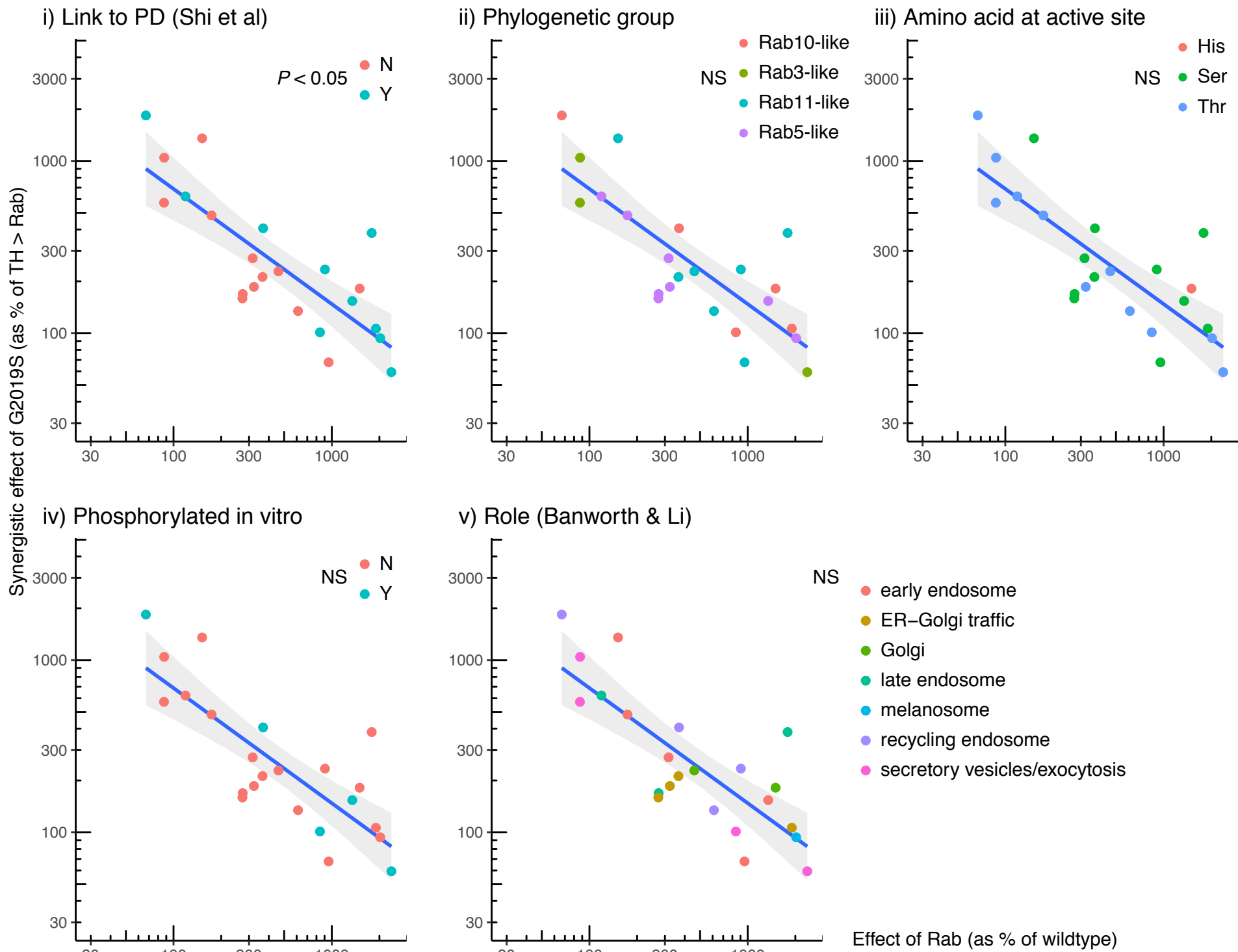
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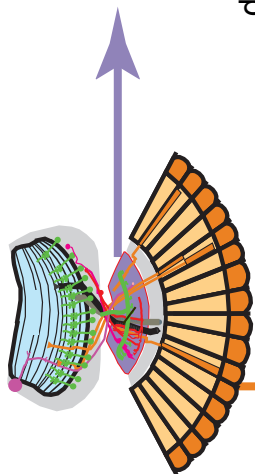


A

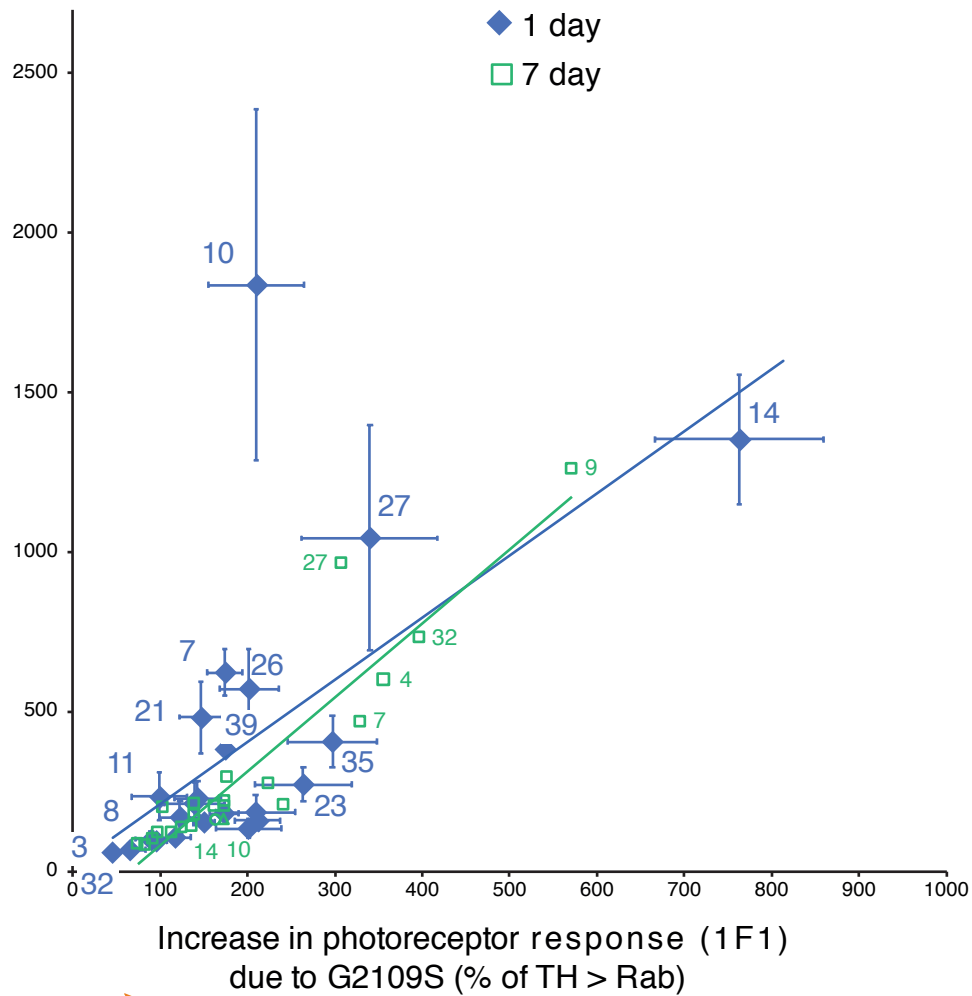


B

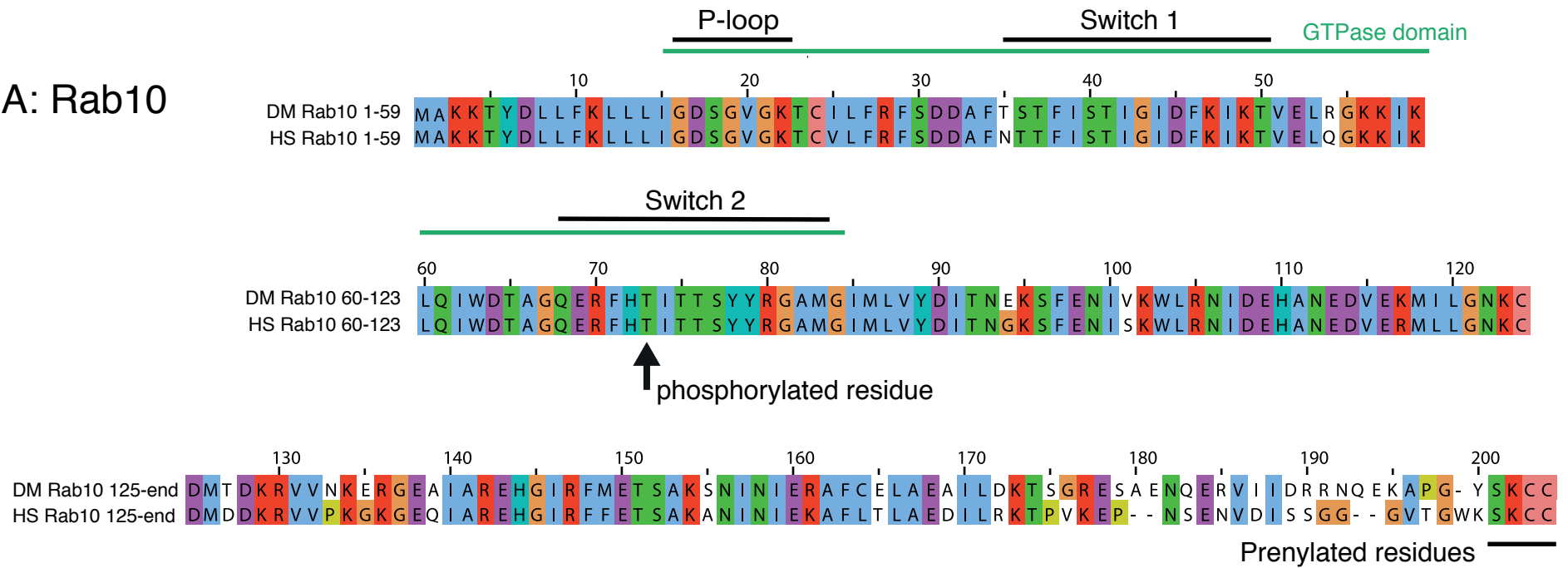




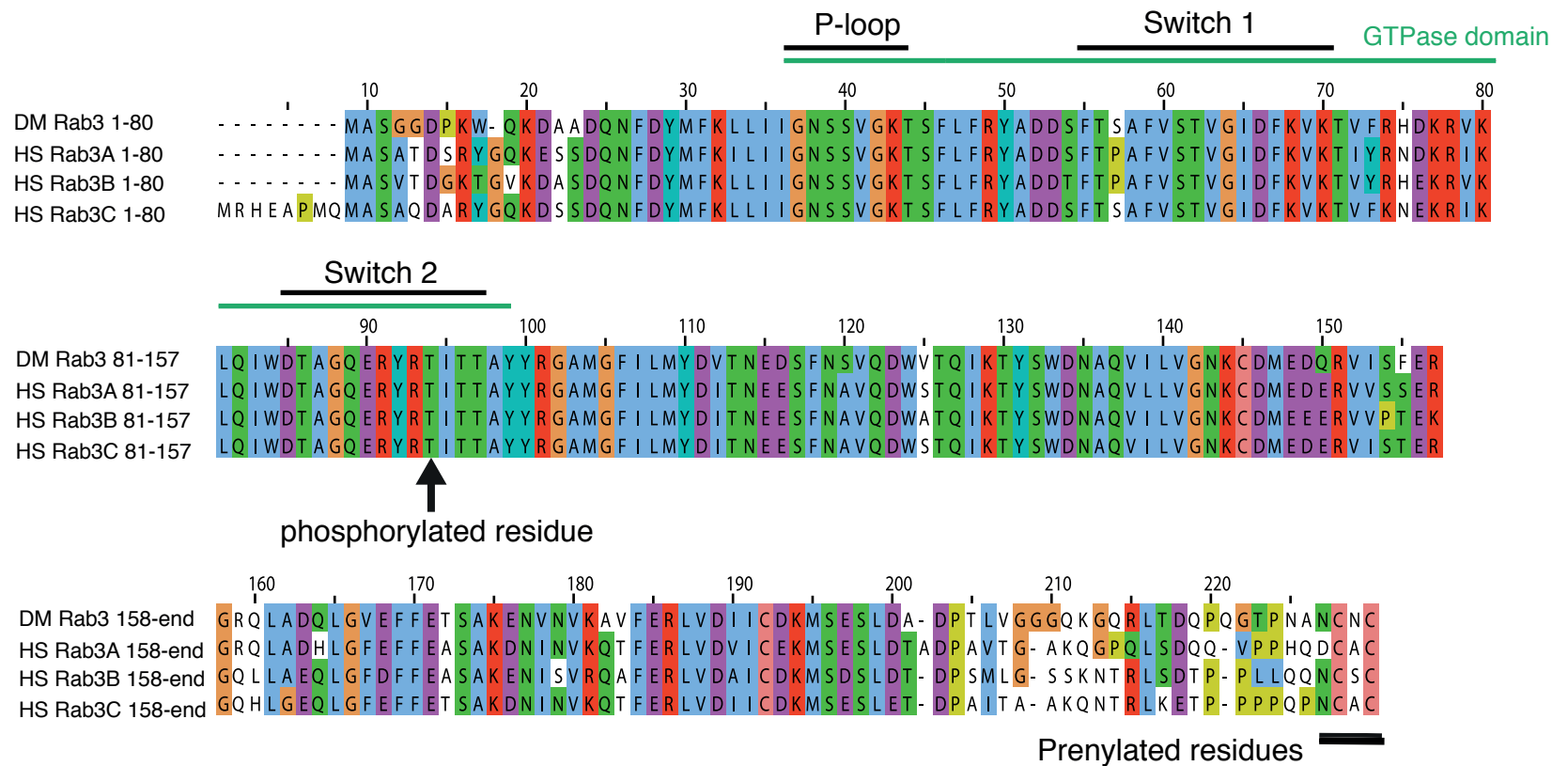
Increase in neuronal response
due to G2109S (as % of TH > Rab)



A: Rab10

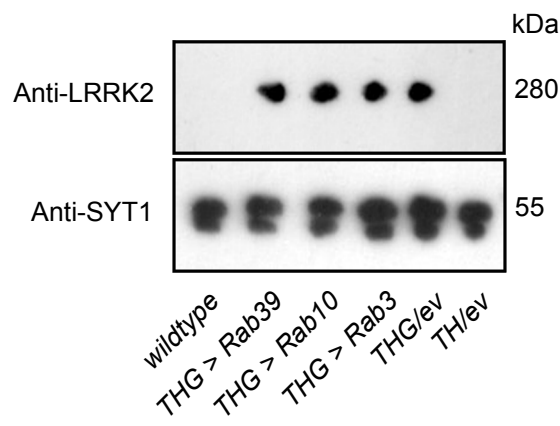


B: Rab3

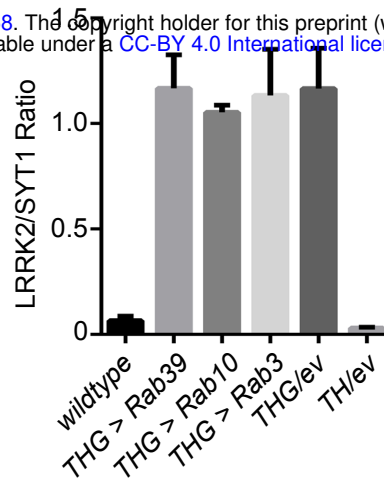


A i

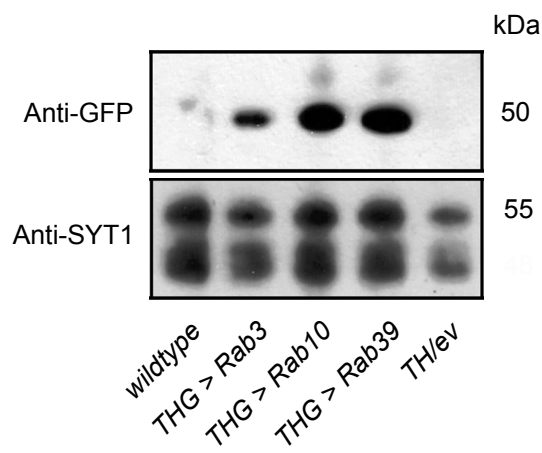
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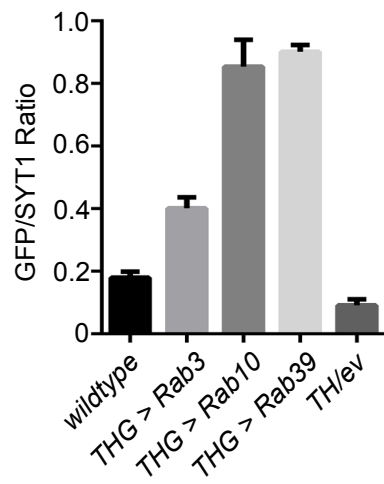
ii



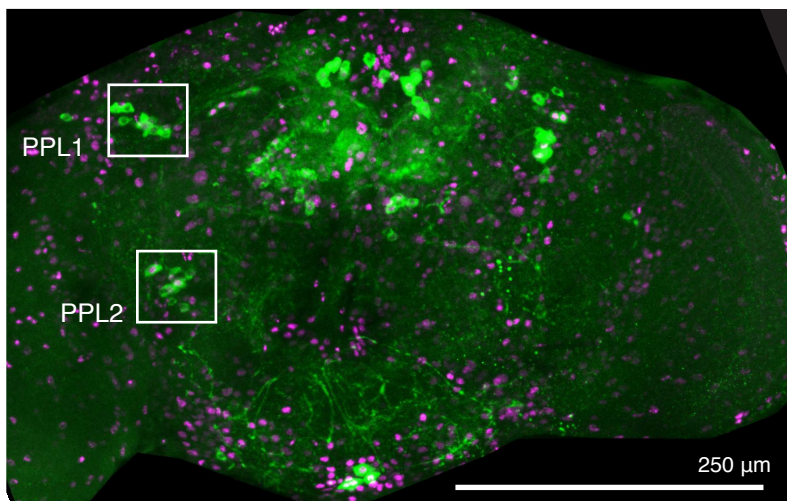
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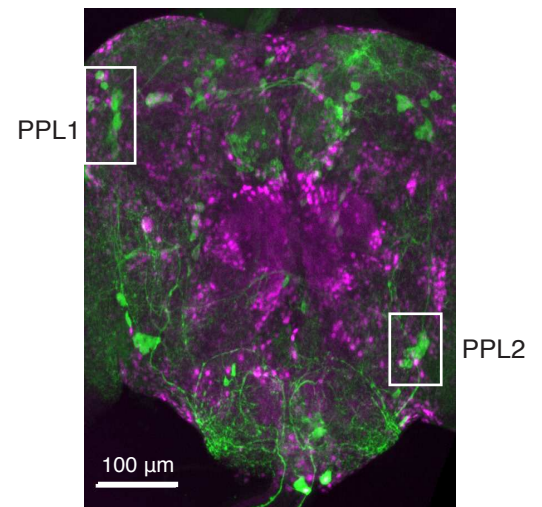
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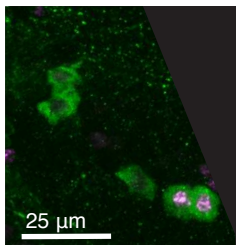
i whole CNS



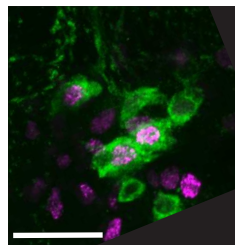
i whole CNS



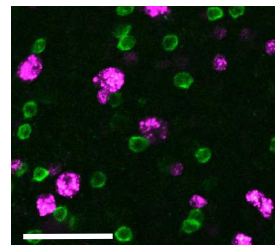
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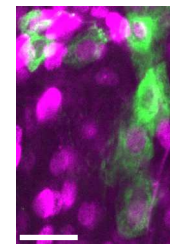
iii PPL2



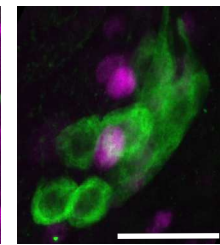
iv MC



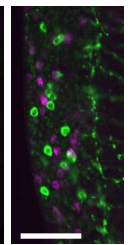
ii PPL1



iii PPL2

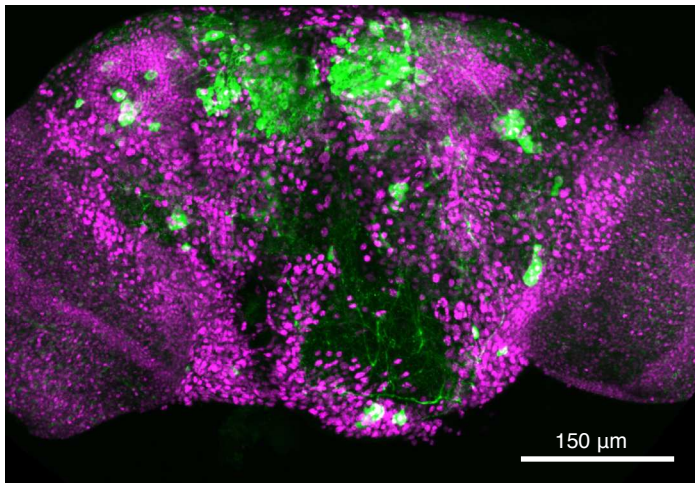


iv MC



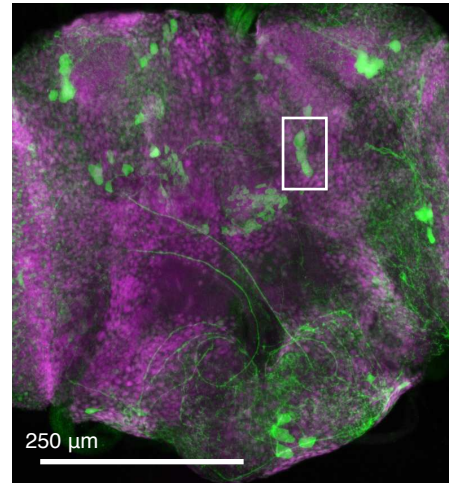
C Rab3 > nRFP; α-TH

whole CNS

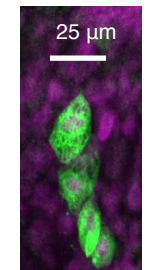


D Rab3 > elf-GFP; α-TH

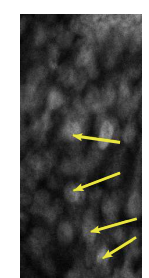
i whole CNS



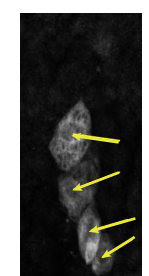
ii PPL1



iii elf-GFP

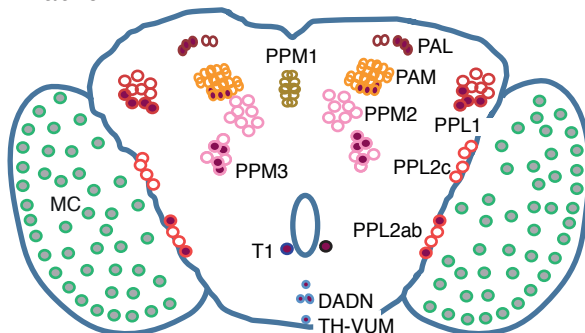


iv α-TH

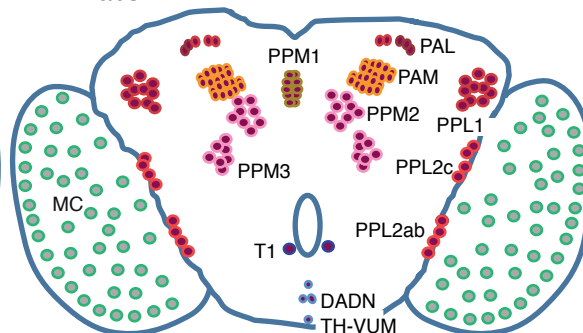


E Summary

i Rab10



ii Rab3



● detected ○ undetectable ◐ undetermined

